METHOD DEVELOPMENT AND VALIDATION OF INDAPAMIDE AND NEBIVOLOL HYDROCHLORIDE IN TABLET DOSAGE FORM BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Dissertation Submitted to

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In partial fulfillment of the requirements for the award of the degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

Submitted by

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MAY- 2012.

CERTIFICATE

This is to certify that the dissertation entitled **"METHOD DEVELOPMENT AND VALIDATION OF INDAPAMIDE AND NEBIVOLOL HYDROCHLORIDE IN TABLET DOSAGE FORM BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY''** is a bonafide and genuine research work carried out at Department of Pharmaceutical Analysis, K.K. College of Pharmacy, Chennai – 600122, by **Ms.V.SATHIYA** during the academic year 2011-2012 under my direct guidance and supervision. This dissertation submitted in partial fulfillment for the award of **Degree of Master of Pharmacy (Pharmaceutical Analysis)** to The Tamil Nadu Dr. M.G.R Medical University, Chennai – 600032.

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V.SATHIYA

LIST OF ABBREVATION

AR grade	- Analytical grade
ºC	- Degree Celcius
Conc	-concentration
GMP	- Good Manufacturing Practice
gm	-Grams
HPLC	- High Performance Liquid Chromatography
ICH	- International Conference on Harmonisation
IEC	- International Electrotechnical Commission
ISO	- International Organization For Standardization
µg	- Microgram
µl	- Microliter
mg	- Milligram
ml	- Milliliter
mV	-millivolt
nm	- nanometer
%	- Percentage
pH	-Negative Logarithm of Hydrogen Ion
RP-HPLC	- Reverse Phase High Performance Liquid Chromatography
RSD	- Relative Standard Deviation
SD	- Standard Deviation
USFDA	- United States Food And Drug Administration
UV	- Ultra Violet Spectroscopy
v/v	- Volume/Volume
w/w	- Weight/weight

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I. INTRODUCTION

Analytical chemistry is the study of the chemical composition of natural and artificial materials that deals with the separation, identification and determination of components in a sample. In other words, it is the art and science of determining what matter is and how much of it exists , which requires background knowledge of chemical and physical concepts.

Analytical chemistry ia a measurement science consisting of a set of powerful ideas and methods that are useful in all fields of science and medicine. Hence ,it plays a vital role in the development of science.

Pharmaceutical analysis plays a major role today, derives its principles from various branches of science like chemistry, physics, electronics, nuclear science etc. Analytical method used for science of sampling, defining, isolating, concentrating, set error limits validate and verify results through calibration and standardization. Analytical method is a specific application of a technique to solve an analytical problem.

Analytical instrumentation plays an important role in the production and evaluation of new products and in the protection of consumers and the environment. This instrumentation provides the lower detection limits required to assure safe foods, drugs, water and air. Analytical chemistry has been split into two main types,

Qualitative analysis- identifying functional groups or elements, or information regarding the presence or absence of one or more components of the sample taken.

Quantitative analysis- amount of analyte present in the sample such as mass or volume is determined using appropriate analytical method.

Instrumental methods of analysis:

The newer methods for separating and determining chemical species are collectively as instrumental methods of analysis. Most of the instrumental techniques fit in to one of the three principle areas such as

- Spectroscopic Techniques
- Chromatographical Techniques
- Electrochemical Techniques
- Hyphenated Techniques
- Miscellaneous Techniques

Spectroscopic techniques:

Spectroscopy is the branch of science dealing with the study of interaction of electromagnetic radiation with matter. The most important consequence of such interaction is that energy is absorbed, scattered or emitted by the matter in discrete amounts called quanta.

Examples:

- Ultra-violet spectrophotometry
- nuclear magnetic resonance spectrometry
- atomic spectrometry
- infrared spectrometry
- X-Ray spectrometry
- Raman spectrometry

Chromatography techniques:

Chromatography is a technique which separates components in a mixture due to the differing time taken for each component to travel through a stationary phase when carried through it by a mobile phase.

Examples:

- Gas chromatography
- High performance liquid chromatography
- Super critical-fluid chromatography

Electrochemical techniques:

Electroanalytical methods measure the potential(volts) or current (amps) in an cell electrochemical containing analyte.

Examples:

- Potentiometric techniques
- Coulometry techniques
- Voltametric techniques
- Amperomeric techniques
- Electrogravimetry

Hyphenated techniques:

Hyphenated techniques combine chromatographic and spectral methods to exploit the advantages of both.

Examples:

- GC-MS (Gas Chromatography – Mass Spectrometry)
- GC-IR (Gas Chromatography – Infrared Spectroscopy)
- MS-MS (Mass Spectrometry – Mass Spectrometry)
- ICP-MS (Inductivity Coupled Plasma Mass Spectrometry)

Miscellaneous techniques:

Examples :

- Thermal analysis
- Mass spectrometry
- Kinetic techniques

CHROMATOGRAPHY[19]

A Russian botanist , Mikhail tswett(1872-1919) was credited with the first use chromatography in 1906 when he separated plant pigments such as chlorophylls and xanthophylls. he passed them through a glass column packed with calcium carbonate. These pigments are coloured and thus the technique was named using the greek terms 'chroma' meaning 'colour' and 'graphein' meaning to write chromatography.

Chromatography is the collective term for the separation of mixtures. The mixture is dissolved in a fluid called the mobile phase which carries it through a structure holding another material called the stationary phase. The various constituents of mixture travel at different speeds, causing them to separate. The separation is based on differential partitioning between the mobile and the stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation.

CLASSIFICATION OF CHROMATOGRPHY:

Based on the principles Involved:

- Absorption Chromatography
- Partition Chromatography
- Ion-exchange Chromatography
- Size exclusion Chromatography
- Affinity Chromatography
- Electrophoretic Chromatography
- Chiral chromatography

Types of Chromatography:

Chromatography characterized as a separation method based on the differential migration of solute through a system of two phases, one is mobile phase another one is stationary phase.

Chromatography is a technique by which the components in a sample, carried by the liquid or gaseous phase, are resolved by sorption, desorption steps on the stationary phase.

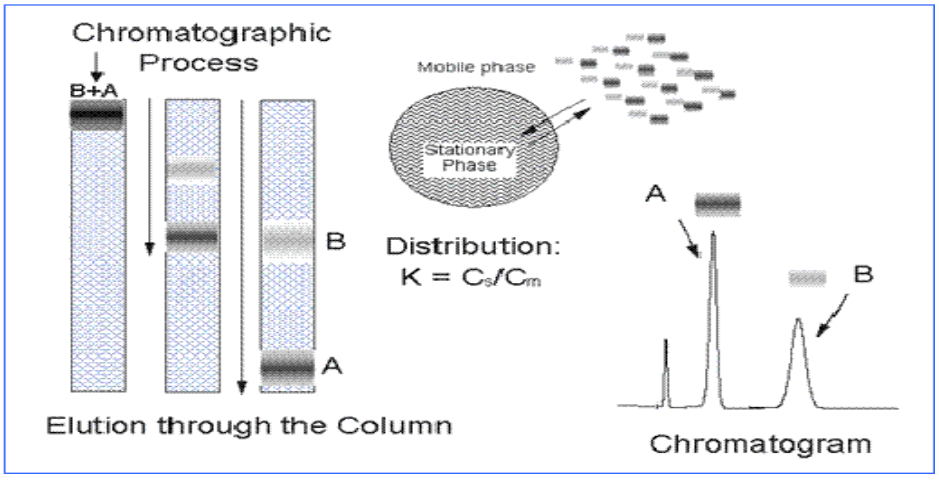
Table no-1-classification of chromatography

General Classification	**Specific Method Name**	**Stationary Phase**	**Type of Equilibrium**
Liquid Chromatography (LC) Mobile Phase = liquid	Liquid-liquid or partition	Liquid adsorbed on a solid	Partition between immiscible liquids
	Liquid-bonded phase	Organic species bonded to a solid surface	Partition between liquid and a bonded surface
	Liquid-solid or adsorption	Solid	Adsorption
	Ion exchange	Ion-exchange resin	Ion exchange
	Size exclusion	Liquid in interstices of a polymeric solid	Partition/sieving
Gas Chromatography (GC) Mobile Phase = gas	Gas-liquid	Liquid adsorbed on a solid	Partition between gas and liquid
	Gas-bonded phase	Organic species bonded to a solid surface	Partition between liquid and bonded surface
	Gas-solid	Solid	Adsorption

HIGH PERFORMANCE LIQIUD CHROMATOGRAPHY

The analytical technique of high performance liquid chromatography is used extensively throughout the pharmaceutical industry. It is used to provide information on the composition of drug related samples. High Performance Liquid Chromatography (HPLC) is one mode of chromatography, one of the most used analytical techniques. Chromatographic process can be defined as separation technique involving mass-transfer between stationary and mobile phase. HPLC utilises a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a shigh pressure. In the column, the mixture separates into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures.

Process of separation of HPLC



HPLC separation is based on interactions and differential partition of sample between the mobile phase and stationary phase.

TYPES OF HPLC:

Based on Modes of Chromatography:

- Normal phase chromatography
- Reverse phase chromatography

Based on Principle of Separation:

- Adsorption chromatography
- Partition chromatography
- Ion exchange chromatography
- Size exclusion chromatography
- Affinity chromatography
- Chiral phase chromatography

Base on Elution Technique:

- Isocratic separation
- Gradient separation

Based on the Scale of Operation:

- Analytical HPLC
- Preparative HPLC

Normal phase chromatography:

Normal phase chromatography is chromatographic technique that uses organic solvents for mobile phase and a polar stationary phase

Reverse phase chromatograghy:

Modifying the polar nature of silanol group by chemically reacting silica with organic silanes. The objective was to make less polar or non polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the chemically modified

silica is now reversed i.e. it is non-polar or the nature of the phase is reversed. The chromatographic separation carried out with such silica is referred to as reversed- phase chromatography.

The term reversed-phase describes the chromatography mode that is just the opposite of normal phase, namely the use of a polar mobile phase and a non-polar [hydrophobic] stationary phase.Today, because it is more reproducible and has broad applicability, reversed-phase chromatography is used for approximately 75% of all HPLC methods. Most of these protocols use as the mobile phase an aqueous blend of water with a miscible, polar organic solvent, such as acetonitrile or methanol. This typically ensures the proper interaction of analytes with the non-polar, hydrophobic particle surface. A C18–bonded silica [sometimes called ODS] is the most popular type of reversed-phase HPLC packing.

Adsorption chromatography:

Adsorption chromatography is one of the oldest types of chromatography around. It utilizes a mobile liquid or gaseous phase that is adsorbed onto the surface of a stationary solid phase. The equilibration between the mobile and stationary phase accounts for the separation of different solutes.

Compounds such as silica acid(silica gel), aluminium oxide, calcium carbonate, magnesium carbonate, magnesium oxide and cellulose may be used as stationary phase, the choice of any particular adsorbent and solvent elution system is dependent on the separation to be achieved.

Partition chromatography:

This form of chromatography is based on a thin film formed on the surface of a solid support by a liquid stationary phase. Solute equilibrates between the mobile phase and the stationary liquid. Mobile phase may be either a liquid or a gas.

The stationary solid surface is coated with a 2nd liquid (the Stationary Phase) which is immiscible in the solvent (Mobile) phase. Partitioning of the sample between two phases delays or retains some components more than others to effect separation**.**

Ion exchange chromatography:

The stationary bed has an ionically charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stationary phase is an ion exchange resin, and separations are governed by the strength of the interactions between solute ions and the exchange sites on the resin. The stronger the charge on the sample, the stronger it will be attracted to the ionic surface and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time.

Size exclusion chromatography:

It is also known as gel permeation or filtration, separation is based on the molecular size or hydrodynamic volume of the components. Molecules that are too large for the pores of the porous packing material on the column elute first, small molecules that enter the pores elute last, and the elution rates of the rest depend on their relative sizes.

Ion-pair/affinity chromatograghy:

Separation is based on a chemical interaction specific to the target species. The more popular reversed phase mode uses a buffer and an added counter-ion of opposite charge to the sample with separation being influenced by pH, ionic strength, temperature, concentration of and type of organic co-solvent(s). Affinity chromatography, common for macromolecules, employs a ligand (biologically active molecule bonded covalently to the solid matrix)which interacts with its homologous antigen (analyte) as a reversible complex that can be eluted by changing buffer conditions.

Chiral chromatography:

Separation of the enantiomers can be achieved on chiral stationary phases by formation of diastereomers via derivatizing agents or mobile phase additives on a chiral stationary phases. When used as an impurity test method, the sensitivity is enhanced if the enantiomeric impurity elutes before the enantiomeric drug.

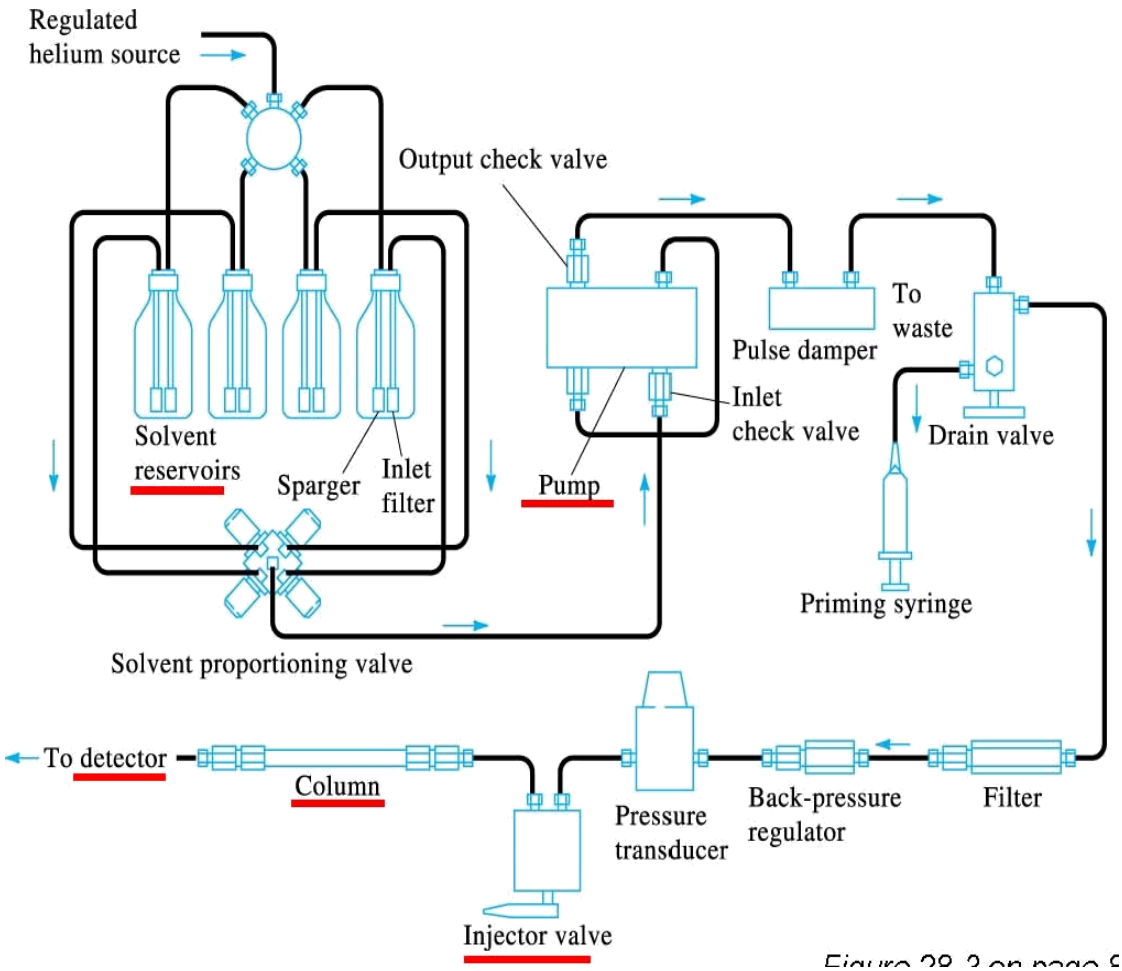
The S-(+) ketamine form is more potent than the R-(-) form. In addition the R-(-) enantiomer may cause post-operative effects. Chromatographic techniques provide fast and accurate methods for enantiomeric separation and allow quantitation using appropriate detection devices. These methods are considered to be most useful for chiral separations. These two approaches are:

Indirect – which utilizes derivatizing agents

Direct- this uses chiral stationary phases or chiral mobile phase additives.

HPLC INSTRUMENTATION:

Schematic diagram of high performance liquid chromatography



INSTRUMENTATION:

The essential of the high performance liquid chromatography are:

- Solvent reservoir
- Mobile phase
- Pump system
- Sample Injection System
- Column
- Detector

Solvent reservoir:

An HPLC system begins with the solvent reservoir made up of glass or stainless steel which contains the solvent used to carry the sample through the system. The reservoir is often equipped with an online degasser which removes the dissolved gases usually oxygen and nitrogen, which interfere by forming bubbles. The solvent should be filtered with an inlet solvent filter to remove any particles which damage the system. Degasser may consist of vaccum pumping system, distillation system, system devices for heating and solvent stirrer.

Mobile phase:

The mobile phase as indicated is the moving phase. Either the mobile or stationary phase is polar and the other is Non-polar. A common polar phase is Methanol, and .non-polar is hexane. The mobile phase carries the mixture through the column stationary phase. The solute can interact with the mobile phase as well as with the stationary phase. Sufficient solubility of solute molecules in the mobile phase must be ensured in order to prevent precipitation.

Pumping system:

HPLC pumping system are required to deliver metered amounts of mobile phase at a constant flow rate. Pumping system that deliver solvent from one or more reservoirs are available. Generating pressure from 6000 psi to 10000 psi pulse free output. Flow rates from 0.1 to 10 ml/min. HPLC pump can be classified in to the following groups according to the manner in which they operate.

Constant displacement pump:

- Reciprocating piston pump
- Syringe drive pump

Constant pressure pump:

- simple gas displacement pump
- pneumatic amplifier pump

Constant displacement pumps:

Constant displacement pumps maintains a constant flow rate through the column irrespective of changing conditions within the column. One form of constant displacement pump is a motor-driven syringe type pump where a fixed volume of solvent is forced from the pump to the column by a piston driven by a motor. Such pumps as well as providing uniform solvent flow rates, also yields a pulse less solvent flow which is important as certain detectors are sensitive to changes in solvent flow rate.

Reciprocating pumps:

Reciprocating pump is most commonly used form of constant displacement pump. The piston moved by a motorized crank, and entry of solvent from reservoir to the pump chamber and exit of solvent to the column is regulated by check valves. On the compression stroke solvent is forced from the pump chamber into the column. During the return stroke the exit check valve closes and solvent is drawn in via the entry valve to the pump chamber, ready to be pumped onto the column on the next compression stroke. Such pumps produce pulses of flow and pulse of flow and pulse dampeners are usually incorporated into the system to minimizing this pulsing effect.

Constant pressure pump:

Constant pressure pumps produce a pulse less flow through the column, but any decrease in the permeability of the column will result in lower flow rates for which the pump, and the gas in turn forces the solvent from the pump chamber into column. The use of an intermediate

solvent between the gas and the eluting solvent reduces the chances of dissolved gas directly entering the eluting solvent and causing problems during the analysis.

Pneumatic pumps:

Pneumatic pumps are the simplest where a the mobile phase is pushed out of the mobile phase container by the pressure of a pressurized gas. The flow is dependent on the back pressure of the column and usually the flow is limited to pressures below2000 psi.

Sample injection system:

In liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in appropriate solvent. In HPLC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors. With these devices, sample is first transferred at atmospheric pressure from a syringe into a sample loop. Turning the valve from load to inject position connects the sample loop are transferred on to the column. A variety of loop volumes is available, commonly 10-50 µl.

Columns:

The columns used for HPLC are generally made up 316 grade of stainless steel and are manufactured so that they can withstand pressures of 5.5×10^7 pa (8000 psi) with 6.35 mm in external diameter, 4.6 mm in internal diameter and 25 cm long. The best columns are precision bored with an internal mirror finish which allows efficient packing of the column. Porous plugs of stainless steel or Teflon are used in the ends of the columns to retain the packing material. The plug must be homogenous to ensure uniform flow of solvents through the column. Materials other than stainless steel that are used for columns include glass, glass lined steel tube or other inert plastics.

Analytical column- length of the column ranges from 5-30 cm and inner diameter 4 to 5 mm. particle size of packing is 5 to 10 µm.

Preparative column- length of the column 25 cm long and 2-5 diameter with 15-100µm.

Guard column-

The guard column is effective preventing the analytical column from being alternated by those impurities. The guard column is a smaller version of the analytical column; packed with the same type of gels as that is in analytical column. The guard column is placed before the analytical column, thus any potential impurities that may cause damages is trapped inside the guard column before it reaches the analytical column.

DETECTORS:

HPLC detectors pass a beam of light through a column effluent as the fluid passes through a low-volume flow cell. Variation in light are recorded and a chromatograph is generated.

LC detectors are basically of two types

1. Bulk property detectors:

It compares overall changes in a physical property of the mobile phase with and without an eluting solute. e.g. refractive index, dielectric constant or density.

2. Solute property detectors:

It responds to a physical property of the solute which is not exhibited by the pure mobile phase. e.g. UV absorbance, fluorescence or diffusion current. Such detectors are about 1000 times more sensitive giving a detectable signal for a few nanograms of sample.

Some types of high performance liquid chromatography detectors are used in analysis mostly are UV detectors, Fluorescence detectors etc

Hplc detectors:

- UV-Visible absorbance detector (UV-VIS)
- Fluorescence detector
- Refractive Index (RI)
- Electrochemical (ECD)
- UV-Visible absorbance detector (UV-VIS)
- Photo-diode array detector (PDA)
- Conductometric detector
- Mass detectors (MS)
- Radiochemical detection
- Chiral detector (Polarimetric & circular dichrosim)

UV-Visible absorbance detector (UV-VIS):

UV detectors function on tha capacity of may compounds to absorb light in the wavelength range 1880 to 350 nm. The sensor cell usually consist of cylindrical cavity light from a UV light sources passes through the sensor on to a photoelectric cell, the output from which is modified and presented on a potentiometric recorder, a computer screen, or printer. By interposing a monochromater between the light source and the cell, light of a specific wavelength can be selected for detection and thus improve the detector selectivity.

Light scattering detectors nebulize the effluent, vaporize the solvent, and then detect droplets in a light scattering cell.

Electrochemical detectors measure the current from the oxidation/reduction reaction of an analyte at a suitable electrode.

Radiochemical detectors use tritium or carbon-14 to detect the fluorescence associated with beta-particle ionization.

Nuclear magnetic resonance detectors irradiate nuclei that are placed between the poles of a strong magnet. The radiation is absorbed, the parallel nuclei enter a higher energy state, and each atom produces a spectra specific to its location and chemical composition.

Fluorescence detectors that occurs when compounds are excited by shorter wavelength energy and emit higher wavelength radiation.

ANALYTICAL METHOD DEVELOPMENT

Methods need to separate the desired components satisfactory, they need to generate the required results, and they must be reproducible and robust so that they can be used time after the time without problems. Methods are developed for new products when no official methos are available. Alternate methods which are not in pharmacopoeia for existing products are developed to reduce the cost and time for better precision and ruggedness.

Method development steps:

Step-1: Define method objectives and understand the chemistry

Determine the goals for method development (e.g., what is the intended use of the method), and to understand the chemistry of the analyte and the drugs product.

Step-2 : Literature survey

The literature for all types of information related to the analyte is surveyed. For synthesis, physical , chemical properties and relevant analytical methods. Books, periodicals, chemical manufacturers and regulatory agency and other publications are reviewed.

Step-3: Choosing a method

Using the information in the literatures and prints, methodology is adopted. The methods are modified wherever necessary. If there are no prior methods for the analyte in the literature, the compounds that are similar in structure and chemical properties are investigated and worked out.

Step-4: Initial HPLC conditions and optimization

Develop preliminary HPLC conditions to achieve minimally acceptable separations. These HPLC conditions will be used for all subsequent method development experiment.

Step-5: Sample preparation procedure

Determine the appropriate standardization method and the use of relative response factors in calculations.

Step-6: Standardization

Determine the appropriate standardization method and the use of relative response factors in calculations.

Step-7: Final method optimization/robustness

Identify the "weaknesses" of the method and optimize the method through experimental design. Understand the method performance with different conditions, different instrument set ups and different samples.

Step-8:Evaluation of method development with actual samples

The sample solution should lead to unequivocal, absolute identification of the analyte peak of interest apart from all other matrix components.

Step 9: Determination of percent recovery of actual sample and demonstration of quantitative sample analysis

Percent recovery of spiked, authentic standard analyte into a sample matrix that is shown to contain no analyte is determined. Reproducibility of recovery (average +/- standard deviation) from sample to sample and whether recovery has been optimized has been shown. It is not necessary to obtain 100% recovery as long as the results are reproducible and known with a high degree of certainty.

Step 9: method validation

Complete method validation according to ICH guidelines.

Basic criteria for new method development of drug analysis:

- The drug or drug combination may not be official in any pharmacopoeias,

- A proper analytical procedure for the drug may not be available in the literature due to patent regulations.
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients.
- Analytical methods for the quantitation of the drug in biological fluids may not be available.
- Analytical methods for a drug in combination with other drugs may not be available. sThe existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

METHOD VALIDATION:

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice.

Analytical methods need to be validated or revalidated,

- Before their introduction into routine use
- Whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix).
- Whenever the method is changed and the change is outside the original scope of the method.

Method validation has received considerable attention in the literature and from industrial committees and regulatory agencies.

- The U.S. FDA CGMP request in section 211.165 (e) methods to be validated: The accuracy, sensitivity, specificity, and reproducibility of test methods employed by the firm shall be established and documented. Such validation and documentation may be accomplished in accordance with Sec. 211.194(a).

- ISO/IEC 17025 includes a chapter on the validation of methods with a list of nine validation parameters.
- The ICH has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics. ICH also developed a guidance with detailed methodology
- The U.S. EPA prepared a guidance for method's development and validation for the Resource Conservation and Recovery Act (RCRA) .

Parameters of analytical method validation:

- System suitability testing
- Specificity
- Linearity and range
- Accuracy
- Precision
- Limit of detection
- Limit of quantification
- Robustness
- Ruggedness

SELECTIVITY AND SPECIFICITY:

Selectivity is the ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix. **Specificity** for an assay ensures that the signal measured comes from the substance of interest, and that there is no interference from excipient and/or degradation products and/ or impurities.

LINEARITY AND RANGE:

Linearity- This is the method's ability to obtain results which are either directly, or after mathematical transformation proportional to the concentration of the analyte within a given

range. Linearity is determined by calculating the regression line using a mathematical treatment of the results (ie least mean squares) vs analyte concentration.

Range- The range of the method is the interval between the upper and lower levels of an analyte that have been determined with acceptable precision, accuracy and linearity.

ACCURACY:

Accuracy is a measure of the closeness of test results obtained by a method to the true value. Accuracy indicates the deviation between the mean value found and the true value. lt is determined by applying the method to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay. Accuracy is best report as percentage bias, which is calculated from the expression.

$$\%\text{Bias} = \frac{\text{(Measured value – True value)}}{\text{True value}} \times 100$$

PRECISION:

The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample.

Precision is a measure of the reproducibility of the whole analytical method (including sampling, sample preparation and analysis) under normal operating circumstances. Precision is determined by using the method to assay a sample for a sufficient number of times to obtain statistically valid results (ie between 6 - 1 0). The precision is then expressed as the relative standard deviation.

$$\%\text{RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

LIMIT OF DETECTION:

This is the lowest concentration in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. The limit of detection is important for impurity tests and the assays of dosages containing low drug levels and placebos. The limit of detection is generally quoted as the concentration yielding a signal-to-noise ratio of 2:1 and is confirmed by analyzing a number of samples near this value (6) using the following equation. The signal-to-noise ratio (5) is determined by,

$$s = H/h$$

Where H = height of the peak corresponding to the component h = absolute value of the largest noise fluctuation from the baseline of the chromatogram of a blank solution.

The other method for calculating LOD is the standard deviation of the intercept (Sd), which may be related to LOD and the slope of the calibration curve, b

$$LOD = \frac{3.3 \times \text{Standard deviation}}{\text{Slope}}$$

LIMIT OF QUANTIFICATION (LOQ):

This is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.

lt is quoted as the concentration yielding a signal-to-noise ratio of 1 0: 1 and is confirmed by analyzing a number of samples near this value calculate by this formula

$$LOQ = \frac{10 \times \text{Standard deviation}}{\text{Slope}}$$

RUGGEDNESS:

Ruggedness is the degree of reproducibility of results obtained by the analysis of the same sample under a variety of normal test conditions like different analysts, laboratories, instruments, reagents, assay temperatures, small variations in mobile phase, different days etc. (from laboratory to laboratory, from analyst to analyst).

ROBUSTNESS:

Robustness of an analytical method is measure of its capacity to remain unaffected small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Testing varying some or all condition:

-Column temperature

-PH of buffer in mobile phase

-Reagents and flow rate

I.1.OBJECTIVE

The objective of any analytical measurement is to obtain consistent, reliable data. Validated analytical methods play a major role in achieving this goal. The results from method validation can be used to judge the quality, reliability and consistency of analytical results, which is an integral part of any good analytical practice.

Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals.

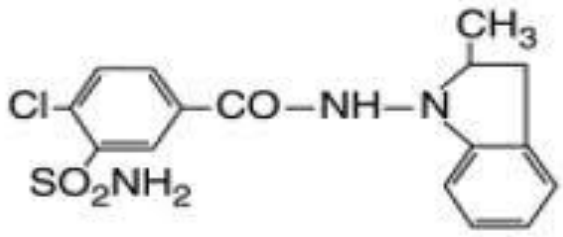
Pharmaceutical products formulated with more than one drug ,typically referred to as combination products ,are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product. Very few methods are available for simultaneous estimation of multiple drug formulations. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical method. Hence, it is essential to develop newer analytical methods which are accurate, linear, precise, specific, linear, simple and rapid. From the literature survey it has been concluded that several methods were reported for estimation of Indapamide and Nebivolol hydrochloride individually and only one or two methods are available for simultaneous estimation of these drugs.

Hence , the present work was thought to develop a precise, accurate, simple and reliable, less time consuming method for simultaneous estimation of indapamide and nebivolol hydrochloride by RPHPLC.

I.2. DRUG PROFILE

INDAPAMIDE

STRUCTURE :



Chemical structure of Indapamide

CHEMICALNAME	**:**	4-chloro-N-(2-methyl-2,3-dihydroindol-1-yl) - 3-sulfomoyl benzamide
MOLECULAR FORMULA	**:**	$C_{16}H_{16}ClN_3O_3S$
MOLECULAR WEIGHT	**:**	365.835g/mol
DESCRIPTION	**:**	Yellow to white coloured powder
CATEGORY	**:**	Thiazide- like(non-thiazide) diuretic drug
DOSE	**:**	1.25mg to 5 mg

Mechanism of action:

Indapamide enhances excretion of sodium ,chloride and water by interfering with the transport of sodium ions across the renal tubular epithelium. It works by preventing the kidney from reabsorbing salt and water that is destined to be eliminated in the urine. This results in increased urine output.

Indapamide also reduce salt in the smooth muscle of the walls of blood vessels. The loss of salt from the muscle causes the muscle to relax and the relaxation of the vessels results in reduced blood pressure.

Indication:

For the treatment of hypertension, alone or in combination with other antihypertensive drugs, as well as for the treatment of salt and fluid retention associated with congestive heart failure lowering high blood pressure helps prevent strokes, heart attacks and kidney problems

.

Pharmacodynamics:

Indapamide is an antihypertensive and a diuretic. It contains both a polar sulfamoyl chlorobenzamide moiety and a lipid-soluble methylindoline moiety. Chemically it does not possess the thiazid e ring system and contains only one sulfonamide group. Indapamide appears to cause vasodilation, probably by inhibiting the passage calcium and other ions like sodium,potassium across membranes.

Pharmacokinetics :

Absorption –rapidly and completely absorbed from GI tract. Peak plasma levels are achieved within 2-2.5 hours

Distribution-Widely distributed,preferentially and reversibly bound to erythrocytes.

Metabolism- Extensively metabolized in the liver.

Excretion-Via urine (60-70% as metabolites,5-7% as unchanged),via faeces(16-23% remaining dose)

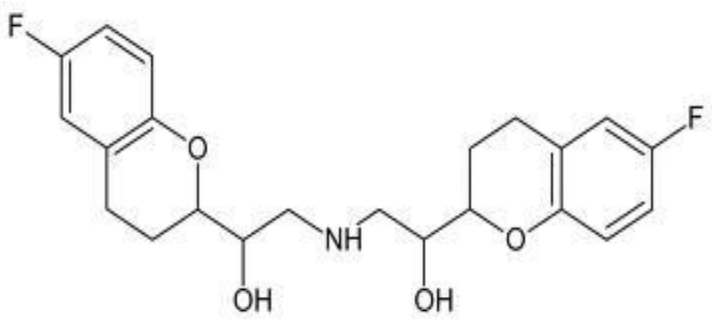
Half life-14 hours

Adverse effects:

- Headache
- Infection
- Anxiety or tension
- Chest pain
- Depression
- Indigestion
- Runny nose
- Sore throat
- Coughing
- Erectile dysfunction

NEBIVOLOL HYDROCHLORIDE

Structure:



. Hcl

Chemical structure of Nebivolol hydrochloride

CHEMICAL NAME **:** 1-(6-fluorochroman-2-yl)-2-[[-2-(6-fluorochroman-2-yl)-2-hydroxyethyl] amino] ethanol hydrochloride

MOLECULAR FORMULA **:** $C_{22}H_{25}F_2NO_4.HCl$

MOLECULAR WEIGHT **:** 441.5g/mol

DESCRIPTION **:** white to off-white powders

CATEGORY **:** β-adrenergic receptor blocking agent

DOSE **:** 2.5 to 20mg

Mechanism of action:

Nebivolol is a β_1 receptor blocker with nitric oxide potentiating vasodilatory effect . In addition beta blockers prevent the release of renin, which is a hormone produced by the kidneys which leads to constriction of blood vessels. However, at doses above 10 mg nebivolol loses its selectivity and blocks both β1 and β2 receptors.

Indication:

Nebivolol hydrochloride used in treatment of hypertension and also treat cardiac failure.

Pharmacodynamics:

II.REVIEW OF LITERATURE

1.)M.J.Legorburu et al[12] (1999) carried out quantitative determination of indapamide in pharmaceuticals and urine by high-performance liquid chromatography with amperometric detection using a µBondapak C_{18} column with mobile phase consists of an acetonitrile: water mixture(45:55 , 5mM) in KH_2PO_4- KH_2PO_4(pH 4.0) and monitered with at + 1200 an amperometric detector equipped with a glassy carbon working electrode. Percentage recovery are 88.3 ± 5.6 and 82.9 ± 7.8. and linearity concentration range from 25 to 315 ng/mL.

2.) LJ Patel et al[3] (2007) developed rphplc and hptlc method for estimation of nebivolol hydrochloride in tablet dosage form. For rphplc method used lichrosper 100 C-18 column with mobile phase buffer(pH3.0±0.1):acetonitrile(45:55v/v) was used. Detection at 282nm and retention time was found to be3.76±0.02 min. For hptlc method linnomat V automatic sampler applicator, Hamilton syringe, camag TLC scanner-3,camag win CAT software with stationary phase precoated silica gel 60F_{254} and mobile phase toluene :methanol: ammonium hydrochloride (1:6:2:0.1v/v/v/v).The detection spot was carried out at 282nm and the R_f value was found at 0.33±0.02. the methods were validated in terms of linearity,accuracy and precsion.

3.)Doshi,Ashish S et al[8] (2008)developed and validated for the determination of nebivolol and valsartan in a capsule formulation by liquid chromatography with mobile phase buffer: acetonitrile (55:45 v/v). The linearity was evaluated over concentration range of 2-8 g/ml and 32-128 g/microg/ml and the accuracy ranged from 100.66 to 102.58% and 101.17 to 101.85% for nebivolol and valsartan.

4.)DA Shah et al[6] (2008) carried out stability indicating RPHPLC method for determination of nebivolol hydrochloride in tablet dosage forms. A phenomax Gemini C-18 with mobile phase buffer(pH-4): methanol: acetonitrile (10:60:30 v/v/v). The retention time was 2.6 min. The linearity was in the range of 0.2-10 µg/ml. the recovery was in the range 98.68-100.86%.

5.)M.K.Sahoo et al[9] (2009) carried out RP-HPLC method for the estimation of nebivolol in tablet dosage form. Chromatography was carried on a hypersil ODS C_{18} column using a mixture of methanol : water(80:20 v/v) as mobile phase at a flow rate of 1.0 ml/min with detection at 282nm.The detector response was linear in the concentration of 1-400 µg/mL. The percentage assay of nebivolol was 99.974%.

6.)M.S.Bhatia et al (2009) carried out simultaneous estimation of nebivolol hydrochloride and valsartan using RP-HPLC. The chromatographic method was standardized using a HIQ sil C-18 column with UV detection at 289 nm and flow rate of 1ml/min.The mobile phase consisting of methanol:water(80:20 v/v) with addition of 0.1 percent 1-hexanesulfonic acid monohydrate sodium salt as an ion-pairing reagent was selected.

7.) Y Gupta et al[4] (2009) developed a new RP-HPLC method for simultaneous estimation of nebivolol hydrochloride and hydrochlorothiazide in pharmaceutical dosage forms using ultra violet detector. Mobile phase consisting of acetonitrile : buffer (pH 3.2 ± 0.1) in the ratio 50:50v/v and flow rate was set on 1.2ml/min at 282 nm. The retention time at 3.57 and 6.66minutes and linear in the range of 20-80mg/ml and 8-32mg/ml for hydrochlorothiazide and nebivolol hydrochloride.

8.)Mishra Vinay Kumar et al (2010) developed simultaneous estimation of amlodipine besylate and nebivolol hydrochloride in combined dosage form by RP-HPLC. The method was carried

out on a Luna C-18, 5µ with a mobile phase consisting of 0.005M ammonium acetate solution , acetonitrile and triethylamine in th ratio 60:40:0.1(v/v) with pH3.0 was adjusted with orthophosphoric acid. Detection was carried out at 269 nm at a flow rate of 1.5 ml/min. The retention time of amlodipine and nebivolol hydrochloride was 3.911 and 5.818 min and linearity was found in the range of 10-30µg/ml.

9.) Amardeep Ankalgi et al (2011) developed RP-HPLC method for estimation of nebivolol in pharmaceutical dosage form. The method was carried out on a Hypersil BDS C-18 consisting of acetonitrile:0.3M potassium dihydrogen phosphate in ratio 50:50 (pH-3 adjusted with orthophosphoric acid) as mobile phase at a flow rate of 1.2ml/ min. Detection was carried out at 278nm. The retention time of nebivolol was 4.34 min.

10.) Paresh U.Patel et al[(1)] (2011) developed two spectroscopic methods for simultaneous estimation of indapamide and nebivolol hydrochloride in combined tablet dosage. The first was simultaneous equations method formation of simultaneous equations at 241.6nm and 281.5nm, linearity concentration range was 2-12?g/ml and 10-50?g/ml for indapamide and nebivolol hydrochloride. The second was Q- analysis method formation of Q-absorbance equation at 274nm and 281.5nm, linearity concentration range was 2-20g/ml for both indapamide and nebivolol hydrochloride.

11.)Pawar Prachi Vasant et al[(7)] (2011) developed and validated for the estimation of atenolol and indapamide in bulk and tablet dosage forms by rphplc.C-18 column with mobile phase methanol: water: diethylamine: glacial acetic acid(70:30:0.12:0.08) in isocratic mode at a flow rate of 1.2ml/min. th e detection at 240nm.The linearity range from 20-100 µg/ml and 1-5 µg/ml for atenolol and indapamide.

12.) A.Sathish Kumar Shetty, Jyoti B.Pai, et al[2] (2011) developed RPHPLC method for quantitative estimation of indapamide in bulk and pharmaceutical dosage forms. Chromatography was performed on a supelco RPC-18 column with mobile phase buffer(pH 3): acetonitrile (60:40) at flow rate 1ml/min, detection at 240 nm. Retention time at 6.76 ± 0.0145 min, linear calibration plots were obtained between 10-100µg/ml.

13.)Hitendra S.Joshi et al[5] (2011) hplc method development and validated for assay of combine dosage form of atenolol and indapamide in commercial tablets. RPHPLC analysis was performed on a phenomenex make Gemini C18 column with mobile phase buffer (pH-3) : acetonitrile (50:50v/v) detection at 241nm. Linearity range 0.2-0.8mg/ml and accuracy was between 98.02 and 100.51%.

14.)S.J. Dighade et al (2011) developed RP-HPLC method for simultaneous estimation of nebivolol hydrochoride and amlodipine besilate in tablet dosage forms. The separation was achieved by C18 intersil column with mobile phase containing methanol : acetonitrile : 50mMkH_2PO_4 buffer (25:30:45 v/v, pH 3.0). UV detection was carried out at 267 nm. The retention time for nebivolol hydrochloride and amlodipine besilate was 7.63 min and 3.34 min and linearity was 10-100 µg/ml for both the drugs.

15.)Patel Amit R et al[10] (2011) developed stability- indicating assay method for simultaneous determination of telmisartan and indapamide in a formulation in the presence of its degradation products and separation from their associated main impurities and degradation products. Amazon C18 column used with mobile phase buffer(pH-3): acetonitrile: methanol(45:25:30) flow rate 1ml/mim and uv detection at 285nm. Retention time at 4.7 min and 10.7 min, linearity in the range of 6-22.5 micro g/ml and 11.2-42 micro g/ml for telmisartan and indapamide.

III. EXPERIMENTAL WORK

METHOD DEVELOPMENT

Instruments used:

Analytical balance - unibloc model ; shimandzu,libror

HPLC - LC-2010 ; shimandzu corporation Japan

UV - UV-2550 ; shimandzu corporation Japan

Chemicals used:

- Water- HPLC grade
- Methanol- HPLC grade
- Acetonitrile- HPLC graded
- Potassium dihydrogen phosphate- AR grade
- Dipotassium hydrogen phosphate- AR grade
- 0.1M sodium hydroxide- AR grade
- Hydrochloric acid- AR grade
- Ortho phosphoric acid- AR grade

Drug sample:

Indapamide, Nebivolol hydrochloride sample obtained from Chandra labs Pvt.Ltd., Hyderabad.

Initialization of the instrument:

Initialy, the column was placed on the instrument and switch on the instruments and washed in methanol: water (20:80) for 30 min. Then the system was made to run with the mobile phase for 30 min.

TRAILS:

1. Trail-I

1.1. Preparation of mobile phase:

Filtered and degassed mixture of acetonitrile:water in the ratio of 65:35 and filtered through 0.45 micron membrane filter.

2. Trail-II

2.1. Preparation of mobile phase:

Filtered and degassed mixture of water:methanol in the ratio of 30:70 and filtered through 0.45 micron membrane filter.

3. Trail-III

3.1. Preparation of Buffer:

Taken 1.1818 gms of potassium dihydrogen phosphate and was dissolved in 500ml of water and adjust the pH-3 using Ortho-phosphoric acid.

3.2. Preparation of mobile phase:

Filtered and degassed mixture of buffer : acetonitrile in the ratio of 35:65 and filtered through 0.45 micron membrane filter.

4. TRAIL-IV

4.1. Preparation of Buffer:

Taken 1.1818gms of potassium dihydrogen phosphate and 0.218gms of Dipotassium hydrogen phosphate was dissolved in 500ml of water and adjusted the pH-3using Ortho-phosphoric acid**.**

4.2. Preparation of mobile phase:

Filtered and degassed mixture of buffer : acetonitrile in the ratio of 40:60 and filtered through 0.45 micron membrane filter.

5. ASSAY

5.1. Preparation of standard solution:

Taken 15 mg of indapamide and 25 mg of nebivolol in 50 ml volumetric flask. Dissolved in methanol and diluted to volume with mobile phase. Transferred 0.5 ml from stock solution to 10 ml volumetric flask and made up to the volume with mobile phase.

5.2. Preparation of sample solution:

Weighed 10 tablets, calculated the average weight , powdered, weighed 500mg and transferred to 50 ml volumetric flask. Dissolved in methanol and diluted to volume with mobile phase, transferred 0.5 ml to 10 ml volumetric flask and made up to the volume with mobile phase.

Calculation:

$$\text{Assay\%} = \frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{Avg.Wt}{\text{Label claim}} \times 100$$

Where, AT = average area counts of sample preparation.

AS= average area counts of standard preparation.

WS = Weight of working standard taken in mg.

P = Percentage purity of working standard

LC = label claim

6. VALIDATION:

6.1 System suitability

6.1.1. Preparation of stock solution:

Taken 15 mg of indapamide and 25 mg of nebivolol hydrochloride in 50 ml volumetric flask. Dissolved in methanol and diluted to volume with mobile phase.

6.1.2. Dilution

Transferred 0.5 ml from stock solution to 10 ml volumetric flask and made up to the volume with mobile phase.

6.2. SPECIFICITY

Check for interference from blank, diluents was used as blank.

6.2.1. Preparation of standard solution:

Taken 15 mg of indapamide and 25 mg of nebivolol in 50 ml volumetric flask. Dissolved in methanol and diluted to volume with mobile phase. Transferred 0.5 ml from stock solution to 10 ml volumetric flask and made up to the volume with mobile phase.

6.2.2 Preparation of sample solution:

Weighed 500mg of powdered sample and transferred to 50 ml volumetric flask. Dissolved in methanol and diluted to volume with mobile phase, transferred 0.5 ml to 10 ml volumetric flask and made up to the volume with mobile phase.

6.2.3. Preparation of stock solution:

Taken 15 mg of indapamide and 25 mg of nebivolol hydrochloride in 50 ml volumetric flask. Dissolved in methanol and diluted to volume with mobile phase.

6.2.4.Tested under stress conditions:

i.)Heated on water bath:

Taken 0.5 ml from the stock solution and transferred to 10 ml volumetric flask, made upto to the volume with mobile phase. The solution should be heated 40^{o}C for 30 minutes. Observed for any degradation occurred.

ii.) Treated with Acids:

Taken 0.5 ml from the stock solution and transferred to a 10 ml volumetric flask. Added1ml of 0.1M hydrochloric acid to that solution and made up to the volume with mobile phase. Observed for any change took place in the retention of the peak.

iii.) Treated with base:

Taken 0.5 ml from the stock solution and transferred to a 10 ml volumetric flask. Added1 ml of 0.1M sodium hydroxide to that solution and made up to to the volume with mobile phase Observed for any degradents occurred.

6.3.LINEARITY

6.3.1. Preparation of stock solution:

Taken 15mg of indapamide and 25 mg of nebivolol in 50 ml volumetric flask. Dissolved in methanol and diluted to volume with mobile phase.

6.3.2. Preparation of linearity solution-I: Transferred 0.1ml from stock solution to 10 ml Volumetric flask and made up with mobile phase(the solution become 3 mcg of indapamide and 5 mcg of nebivolol hydrochloride).

6.3.3. Preparation of linearity solution-II: Transferred 0.2ml from stock solution to 10 ml volumetric flask and made up with mobile phase(the solution become 6 mcg of indapamide and 10 mcg of nebivolol hydrochloride).

6.3.4. Preparation of linearity solution-III: Transferred 0.3ml from stock solution to 10 ml volumetric flask and made up with mobile phase(the solution become 9 mcg of indapamide and 15 mcg of nebivolol hydrochloride).

6.3.5. Preparation of linearity solution-IV: Transferred 0.4ml from stock solution to 10 ml volumetric flask and made up with mobile phase(the solution become 12 mcg of indapamide and 20mcg of nebivolol hydrochloride).

6.3.6. Preparation of linearity solution-V: Transferred 0.5ml from stock solution to 10 ml volumetric flask and made up with mobile phase(the solution become 15 mcg of indapamide and 25mcg of nebivolol hydrochloride).

6.3.7. Preparation of linearity solution-VI: Transferred 0.6ml from stock solution to 10 ml volumetric flask and made up with mobile phase (the solution become 18 mcg of indapamide and 30 mcg of nebivolol hydrochloride).

6.4. ACCURACY:

6.4.1. Preparation of stock solution:

Taken 15 mg of indapamide and 25 mg of nebivolol hydrochloride in 50 ml volumetric flask. Dissolved in methanol and diluted to volume with mobile phase.

6.4.2. Preparation of spiking standard:

Transferred 0.5 ml from stock solution to 10ml volumetric flask made up with mobile phase.

6.4.3. Preparation of Accuracy solution 1: Transferred 0.4ml from stock solution to 10 ml volumetric flask and made up with mobile phase(the solution become 12 mcg of indapamide and 20 mcg of nebivolol hydrochloride) and added 1ml of spiking standard.

6.4.4. Preparation of Accuracy solution 2: Transferred 0.5ml from stock solution to 10 ml volumetric flask and made up with mobile phase(the solution become 15 mcg of indapamide and 25 mcg of nebivolol hydrochloride) and added 1ml of spiking standard.

6.4.5. Preparation of Accuracy solution 3: Transferred 0.6ml from stock solution to 10 ml volumetric flask and made up with mobile phase(the solution become 18 mcg of indapamide and 30 mcg of nebivolol hydrochloride) and added 1ml of spiking standard.

6.5 PRECISION

6.5.1. System precision:

Preparation of standard solution:

Taken 15 mg of indapamide and 25 mg of nebivolol hydrochloride in 50 ml volumetric flask. Dissolved in methanol and diluted to volume with mobile phase. Transferred 0.5 ml from Stock solution to 10 ml volumetric flask and made up to the volume with mobile phase.

6.5.2. Method precision:

Preparation of sample solution:

Weighed 500mg of sample and transferred to 50 ml volumetric flask. Dissolved in methanol and diluted to volume with mobile phase, transferred 0.5ml to 10 ml volumetric flask and made up to the volume with mobile phase.

6.5.3. Intermediate precision (ruggedness):

The intermediate precision was performed by two analyst on different instrument and different day with the solution used for the method precision.

6.6. ROBUSTNESS:

6.6.1. Preparation of stock solution:

Taken 15 mg of indapamide and 25 mg of nebivolol hydrochloride in 50 ml volumetric flask. Dissolved in methanol and diluted to volume with mobile phase.

6.6.2. Dilution:

Transferred 0.5 ml from stock solution to 10ml volumetric flask made up with mobile phase (the solution become 15 mcg of indapamide and 25 mcg of nebivolol hydrochloride).

6.7. LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ):

The LOD and LOQ of the drug were derived by visually or calculating the signal- noise ratio. In this method the LOD and LOQ of the drug were calculated by following equation.

$$LOD = \frac{3.3 \times \text{Standard deviation}}{\text{Slope}}$$

$$LOQ = \frac{10. \times \text{Standard deviation}}{\text{Slope}}$$

IV.RESULTS AND DISCUSSION

1. TRAILS

1.1. Trail-I:

System suitability results for Indapamide and Nebivolol hydrochloride

1.) Tailing factor obtained from trail- I was 1.600 and 1.515

2.) Theoretical plates obtained from trail-I was 1353 and 2378

3.) Resolution obtained from trail-I was 12.593

4.)Retention time obtained from trail-I was 1.8 and 6.4

1.2. Trail-II

System suitability results for Indapamide and Nebivolol hydrochloride

1.) Tailing factor obtained from trail-II was 1.333 and 2.192

2.) Theoretical plates obtained from trail-II was 2372 and 2898

3.) Resolution obtained from trail-II was 18.534

4.) Retention time obtained from trail-II was 1.7 and 10.1

1.3. Trail-III:

System suitability results for Indapamide and Nebivolol hydrochloride

1.) Tailing factor obtained from trail-III was 1.074 and 1.159

2.) Theoretical plates obtained from trail-III was 1551 and 7793

3.) Resolution obtained from trail-III was 17.843

4.) Retention time obtained from trail-III was 1.6 and 9.6.

1.4.Trail-IV:

System suitability results for Indapamide and Nebivolol hydrochloride

1.) Tailing factor obtained from trail-IV was 1.429 and 1.250

2.) Theoretical plates obtained from trail-IV was 6081 and 7394

3.) Resolution obtained from trail-IV was 7.416

4.) Retention time obtained from trail-IV was 2.54 and 3.65

Trails results:

on the evaluation of above system suitability results,

- In trail-I and trail-II the tailing factor should to be reduced.
- In trail-I and trail-III the theoretical plates should to be increased , resolution should to be reduced.
- System suitability parameters of trail-IV were within the satisfactory limits.

Hence trail I,II, III shows variation in system suitability results and affected the method significantly .Trail-IV shows good system suitability results and also within in the limit so the trail – IV was adopted.

Table no-2: OPTIMIZED METHOD PARAMETERS:

PARAMETERS	CONDITIONS
Column(Stationary Phase)	BDS HYPERSIL C18 (4.6 x 150mm, 5 µm
Mobile Phase	Phosphate Buffer(3.0pH):Acetonitrile(40:60)
Flow rate (ml/min)	1
Run time (min)	20
Column temperature(°C)	Ambient
Volume of injection loop (µl)	20
Detection wavelength (nm)	235
Drug RT (min)	2.54,3.65

2. ASSAY

In HPLC ,injected the standard and sample solution prepared as per procedure 5.1 and 5.2 and the results were calculated as per the formula given in assay procedure(5).

Table no-3: Assay results of indapamide

INDAPAMIDE		
Standard Area	1	330.677
	2	333.248
	3	332.291
	Average	332.072
Sample area	1	332.830
	2	329.576
	Average	331.203
Tablet average weight		50.4 mg
Standard weight		15mg
Sample weight		500.9 mg
Label amount		1.5 mg
std.purity		99.86%
Cal.:		**1.503 mg**
	%Assay	**100.21 %**

Table no-4 : Assay results of nebivolol hydrochloride

Nebivolol HCL		
Standard Area	1	808.412
	2	805.524
	3	805.524
	Average	806.486
Sample area	1	805.228
	2	807.116
	Average	806.172
Tablet average weight		50.4 mg
Standard weight		25mg
Sample weight		500.9 mg
Label amount		2.5 mg
Std.purity		99.85%
Cal.:		**2.510 mg**
	%Assay	**100.42 %**

Result :

From the assay studies, it was found that the formulation contains 100.21% of indapamide and 100.42%.of nebivolol hydrochloride.

VALIDATION RESULTS

1. System suitability:

In HPLC, injected the solution prepared as per the procedure 6.1 and recorded the peak response of the chromatogram.

Table no-5 : System suitability results for indapamide

S.No	Parameter	Indapamide
1	RT(min)	2.51
2	Tailing Factor	1.429
3	No. of theoretical plates	6081

Table no- 6 : System suitability results for nebivolol hydrochloride

S.No	Parameter	Nebivolol hydrochloride
1	RT(min)	3.62
2	Tailing Factor	1.250
3	No.of theoretical plates	7394

Result :

On the evaluation of above results it was found that all the system suitability parameters were within the satisfactory limit.

2. SPECIFICITY:

Diluents ,standard preparation and assay were prepared as per the method (6.2) and the solutions were injected into the HPLC and the chromatograms recorded. The retention time were given in the following table,

Table no-7: Specificity results for indapamide and nebivolol hydrochloride

S.No	Solutios	Retention time(min)
1.	Indapamide Standard preparation	2.52
2.	Indapamide assay preparation	2.51
3.	Nebivolol Hcl standard preparation	3.63
4.	Nebivolol Hcl assay preparation	3.62

Table no-8: Specificity results for indapamide and nebivolol hydrochloride under stress conditions

S.No	Stress conditions	Observed Result
1.	Heated on water bath	No degradation occurred
2.	Treated with acids	No change in retention of the peak
3.	Treated with base	No degradents formed

Result :

i.) No peaks should be detected at the retention time of indapamide and nebivolol hydrochloride in the chromatograms of diluents preparation

ii.) From the stress conditions performed, various degradation products were formed and there was no change in the detection of the analyte in the presence of other components.

3. LINEARITY

In HPLC,injected the each concentration of the solution prepared as per the method (6.3) and the results were given in the following tables,

Table no-9: Linearity Results: (for indapamide)

S.No	Linearity Level	Concentration (mcg)	Area (mV.s)
1	I	3	68.577
2	II	6	136.152
3	III	9	189.157
4	IV	12	228.469
5	V	15	284.891
6	VI	18	332.874
Correlation Coefficient			**0.997**

Figure No-1: calibration plot for indapamide:

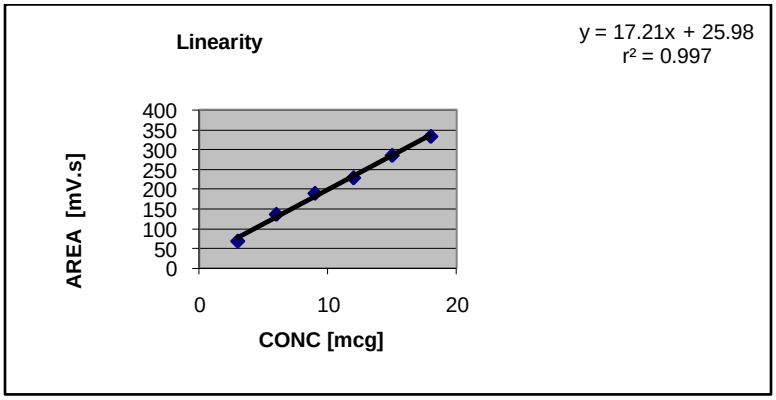
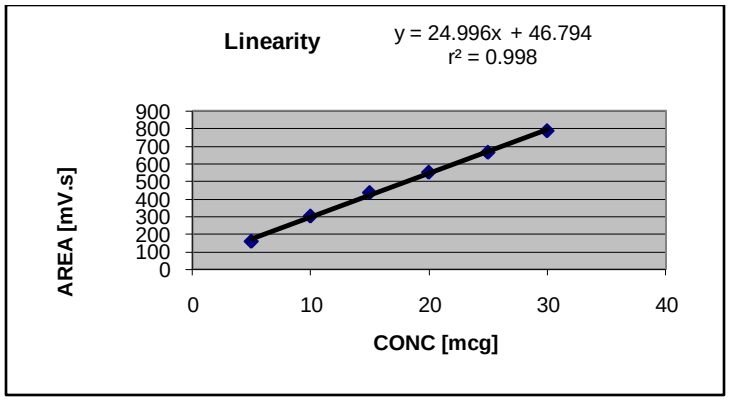


Table no-10: Linearity Results: (for nebivolol hydrochloride)

S.No	Linearity Level	Concentration (mcg)	Area (mV.s)
1	I	5	157.532
2	II	10	301.879
3	III	15	436.291
4	IV	20	552.655
5	V	25	666.779
6	VI	30	790.165
Correlation Coefficient			**0.998**

Figure No:2 calibration plot for nebivolol hydrochloride:



Acceptance criteria:

Correlation coefficient should be not less than 0.9

Results :

On evaluation of above results % RSD values are within the limit hence the curve shows linearity at concentration range 3µg/ml-18µg/ml for indapamide and 5µg/ml-30µg/ml for nebivolol hydrochloride.

4. ACCURACY:

In HPLC, injected each concentration of the solution prepared as per the method(6.4) and the results were given in the following tables,

Table no: 11 Accuracy results for indapamide

(at specification Level)	Area	Amount Added (mcg)	Amount Found (mcg)	% Recovery	Mean Recovery
80%	262.1347	13.5	13.46	99.70%	99.22%
100%	318.4707	16.5	16.31	98.84%	
120%	368.3263	19.5	19.33	99.12%	

Table no-12: Accuracy results of nebivolol hydrochloride:

%Concentration (at specification Level)	Area	Amount Added (mcg)	Amount Found (mcg)	% Recovery	Mean Recovery
80%	637.1297	22.5	22.31	99.16%	99.21%
100%	777.3993	27.5	27.22	98.98%	
120%	883.5633	32.5	32.34	99.50%	

Acceptance Criteria:

The % Recovery for each level should be between 98.0 to 102.0%.

Results :

From the results shown in accuracy, it was found that the percentage recovery values of pure drug from the pre analyzed solutions of formulations were in between 99.22% for indapamide, 99.21% for nebivolol hydrochloride, which indicates that the method was accurate.

5. PRECISION:

In HPLC,injected six replicate injection of the solution prepared as per the method(6.5) and the results were given in the following table,

5.1 System precision:

Table no- 13: System precision results of indapamide

Injections	Area[mv.s]
1	285.986
2	287.305
3	285.395
4	289.254
5	286.513
6.	284.562
Avg	286.5025
Std dev	**1.641569**
%RSD	**0.60**

Table no-14 System precision results for nebivolol hydrochloride

Injections	Area[mV.s]
1	685.131
2	680.701
3	676.947
4	679.709
5	680.747
6	679.988
Avg	680.5372
Std dev	**2.647675**
%RSD	**0.40**

5.2. Method precision:

Table no -15: Method precision results for indapamide

Injections	Area[mV.s]
1	280.641
2	281.961
3	281.057
4	282.752
5	281.340
6	283.343
Avg	281.9502
Std dev	**1.157837**
%RSD	**0.4**

Table no-16: Method precision results for nebivolol hydrochloride

Injections	Area[mV.s]
1	682.656
2	684.543
3	684.116
4	683.652
5	690.639
6	683.454
Avg	684.8433
Std dev	**2.910083**
%RSD	**0.5**

Acceptance Criteria:

The % RSD for the area of five standard injections results should not be more than 2%.

Result :

From the results shown in precision, it was found that % RSD is less than 2%; which indicates that the proposed method has good reproducibility**.**

5.3. Intermediate precision(ruggedness):

Table no-17: Intermediate precision results for indapamide

Injections	**Area [mV.s]**	
	Analyst-1	Analyst-2
1	283.566	283.524
2	280.452	284.433
3	282.421	282.455
4	283.433	282.754
5	281.543	284.653
6	282.233	282.335
Avg	282.2747	283.5257
Std dev	**1.173872**	**0.906894**
%RSD	**0.41**	**0.31**

Table no-18: Intermediate precision results for nebivolol hydrochoride

Injections	**Area [mV.s]**	
	Analyst-1	**Analyst-2**
1	680.323	681.09
2	683.376	683.544
3	683.455	683.423
4	685.431	685.573
5	683.747	685.431
6	684.412	685.443
Avg	683.4573	684.084
Std dev	**1.715886**	**1.764708**
%RSD	**0.30**	**0.31**

6. ROBUSTNESS:

The robustness of the method established by making minor variations in the method parameters like, change in flow rate by ± 10 % of actual flow rate .

Table no-19: Robustness results for indapamide

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	2242	1.5
2	1.0	6081	1.4
3	1.1	3283	1.6

Table no-20: Robustness results for nebivolol hydrochloride

S.No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.9	2804	1.6
2	1.0	7394	1.2
3	1.1	3176	1.6

Result:

The flow rate was varied at 1.0 ml/min to 1.2ml/minThe results are summarized On evaluation of the above results, it can be concluded that the variation in flow rate affected he method significantly. Hence it indicates that the method is not robust even by change in the flow rate ±10%.

7. LIMIT OF DETECTION:

Table no-21:Limit of detection results

Drug Name	Standard Deviation	Slope	Result (µg/ml)
Indapamide	1.641569	17.21	0.30
Nebivolol hydrochloride	2.647675	24.996	0.95

8. LIMIT OF QUANTIFICATION:

Table no-22: Limit of quantification results

Drug Name	Standard Deviation	Slope	Result (µg/ml)
Indapamide	1.641569	17.21	0.31
Nebivolol hydrochloride	2.647675	24.996	1.05

V.SUMMARY AND CONCLUSION

An attempt has been made to develop the RP-HPLC method for simultaneous estimation of indapamide and nebivolol hydrochloride in combined dosage form. As the literature survey revealed that few methods were available for their simultaneous estimation, but there is a need of a simple, economical and proper method for estimation of above combination in combined dosage form.

Water's- HPLC with Empower2 software, UV detector and with BDS HYPERSIL C_{18} Column (4.6mm X 150 mm, 5µm), an injection volume of 20µl is injected and eluted with the mobile phase of phosphate buffer (pH3.0):Acetonitrile (40:60), which was pumped at a flow rate of 1ml/min and detected by UV detector at 235 nm. The peaks of indapamide and nebivolol hydrochloride were found well separated at , 2.54 and 3.65 respectively.

From the assay studies, it was found that the formulation contains 100.12% of indapamide and 100.32%.of nebivolol hydrochloride.

The system suitability studies showed that all the system suitability parameters were within the acceptance criteria and the drug obeys linearity within the concentration range of 3-18µg/ml for indapamide, 5-30µg/ml for nebivolol hydrochloride..

Accuracy results showed that the percentage recovery values of pure drug from the pre analyzed solutions of formulations were in between 99.22% for indapamide, 99.21% for nebivolol hydrochloride, which indicates that the method was accurate and the precision results concludes that % RSD is less than 2%; which indicates that the proposed method has good reproducibility.

From the results shown in Robustness, it was found that there is little change in the results with the change in the parameters like flow rate indicating the robustness of the method and in the ruggedness, the %RSD is less than 2% for the results of two analyst indicating the ruggedness of the method.

The developed chromatographic method for the determination of indapamide and nebivolol hydrochloride in tablet dosage forms was simple, rapid, accurate, precise, specific, robust and economical. Therefore this method may be adopted for the routine analysis of Indapamide and Nebivolol hydrochloride in pharmaceutiacal tablet formulation.

CHROMATOGRAMS

ASSAY CHROMATOGRAMS

Figure no-3:Assay-standard

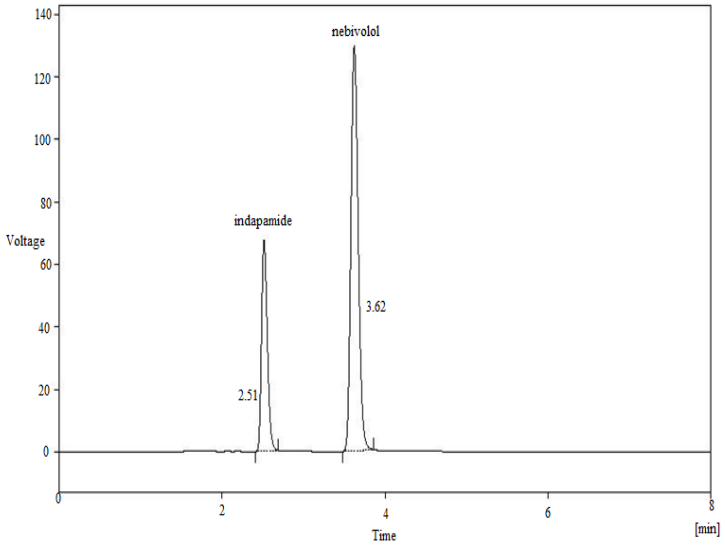
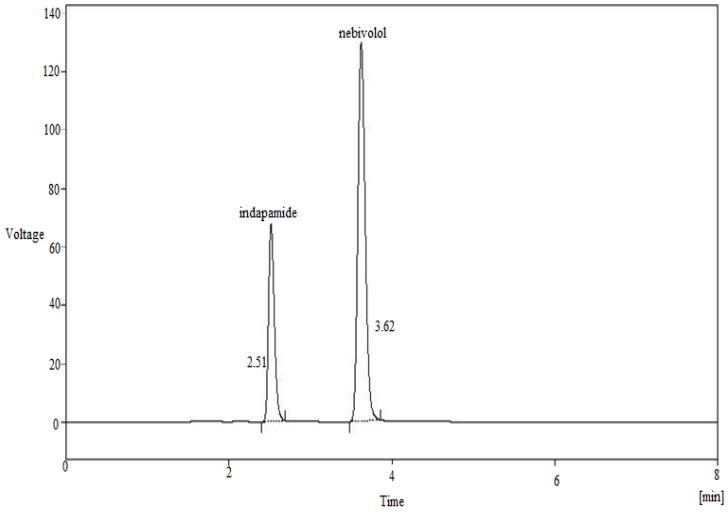


Figure no-4:Assay-sample



SPECIFICITY CHROMATOGRAMS

Figure No-5:Specificity- Diluent (blank)

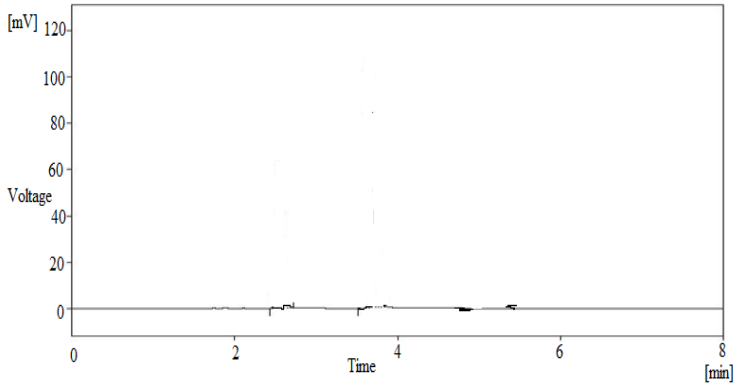


Figure No-6:Specificity- standard solution

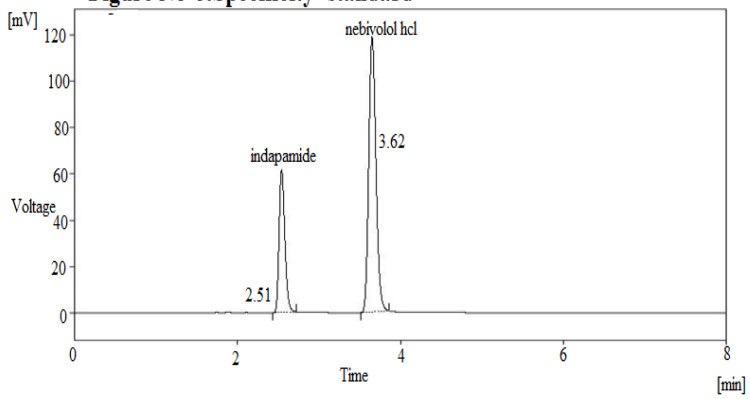


Figure No-7;Specificity sample solution

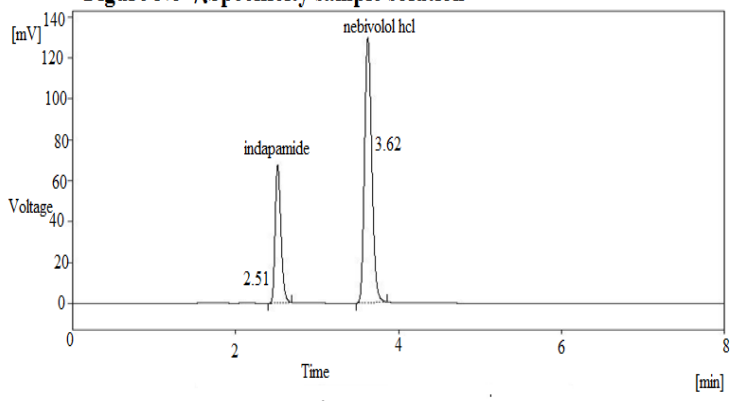


Figure No-8:Specificity under stress condition-heated on water bath

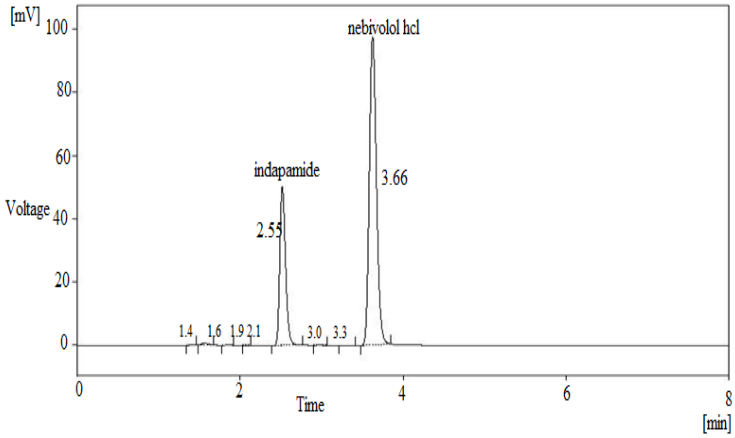


Figure No-9:Specificity under stress condition- treated with acid

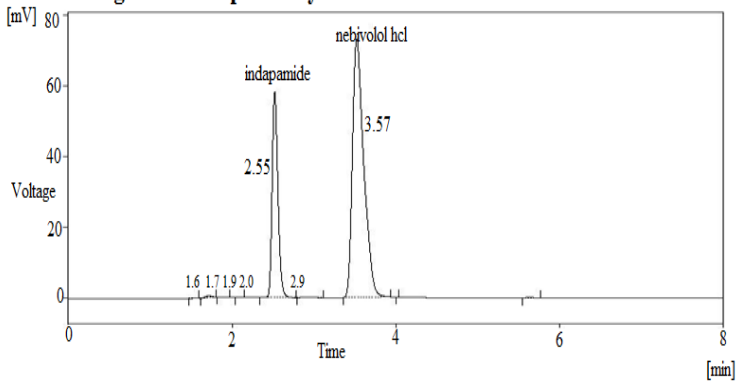
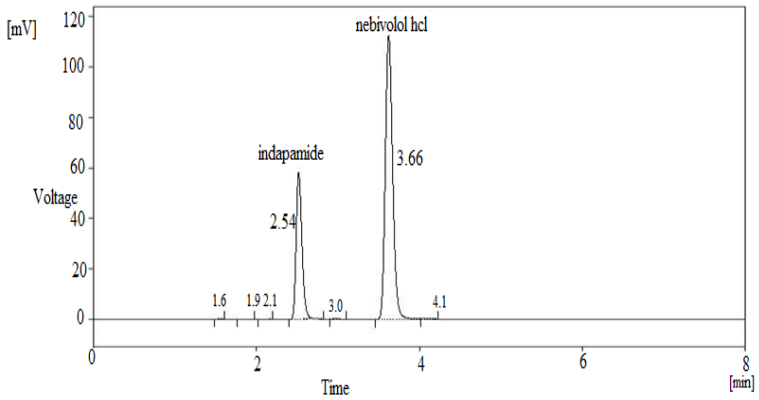


Figure No-10: Specificity under stress condition - treated with base



LINEARITY CHROMATOGRAMS

Figure No-11:Linearity-1[Indapamide(3mcg) and Nebivolol hydrochloride(5mcg)]

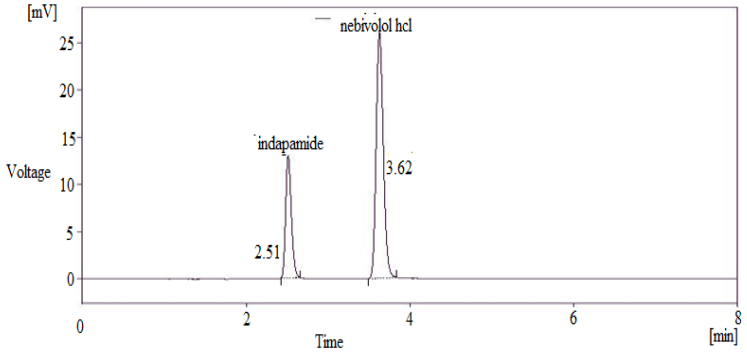


Figure No-12:Linearity-2[Indapamide(6mcg) and Nebivolol hydrochloride(10mcg)]

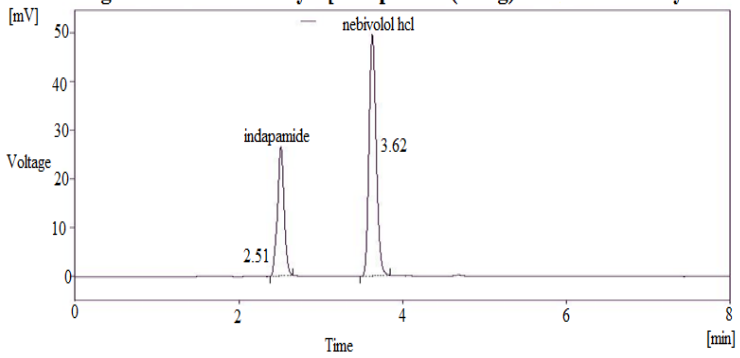


Figure No-13:Linearity-3[Indapamide(9mcg) and Nebivolol hydrochloride(15mcg)]

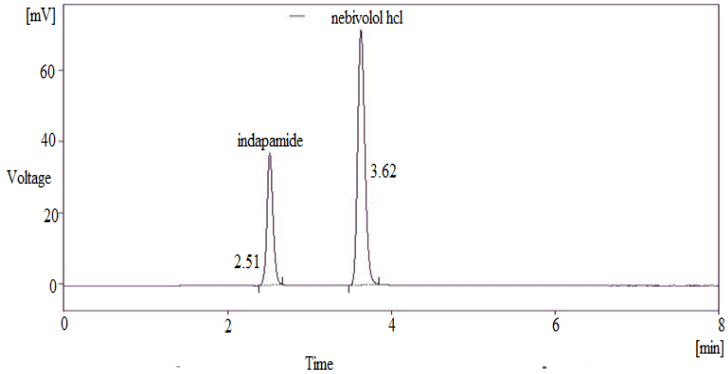


Figure No-14: Linearity-4[Indapamide(12mcg) and Nebivolol hydrochloride(20mcg)]

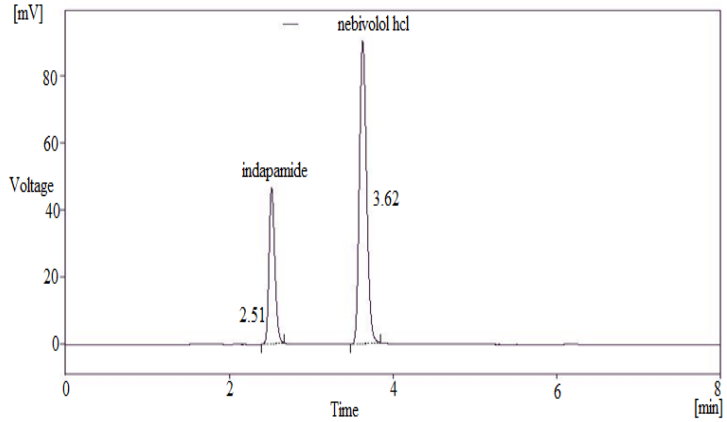


Figure No-15:Linearity-5[Indapamide(15mcg) and Nebivolol hydrochloride(25mcg)]

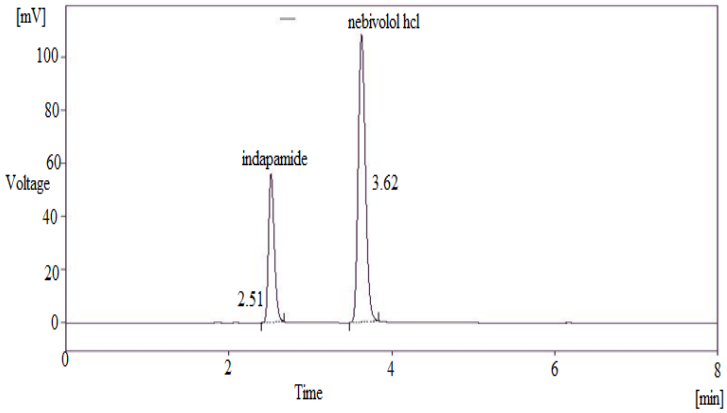
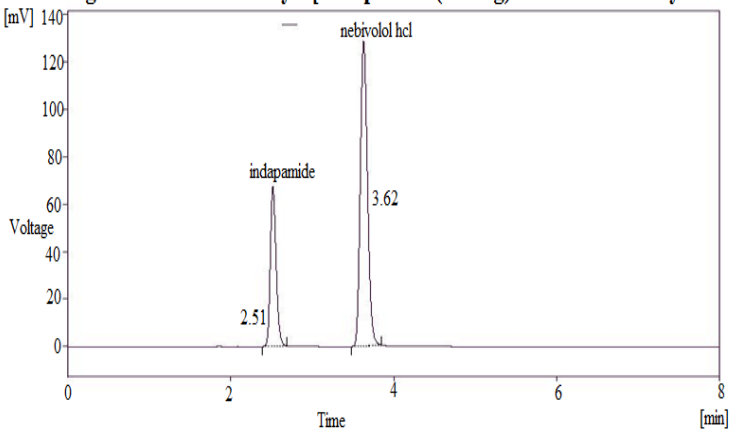


Figure No-16:Linearity-6[Indapamide(18mcg) and Nebivolol hydrochloride(30mcg)]



ACCURACY CHROMATOGRAMS

Figure No-17:Accuracy-80%

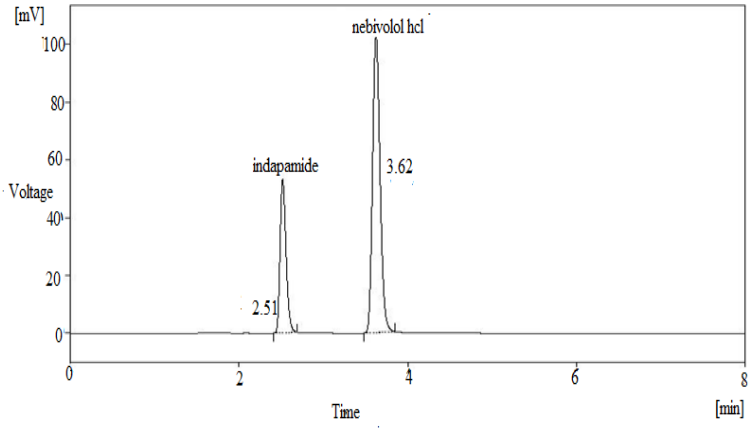


Figure No-18:Accuracy-100%

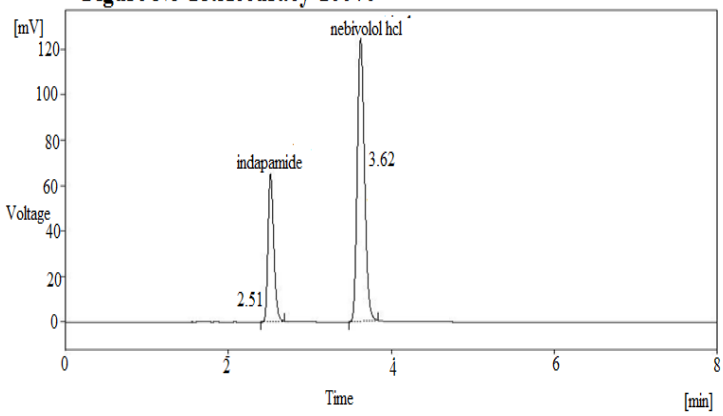
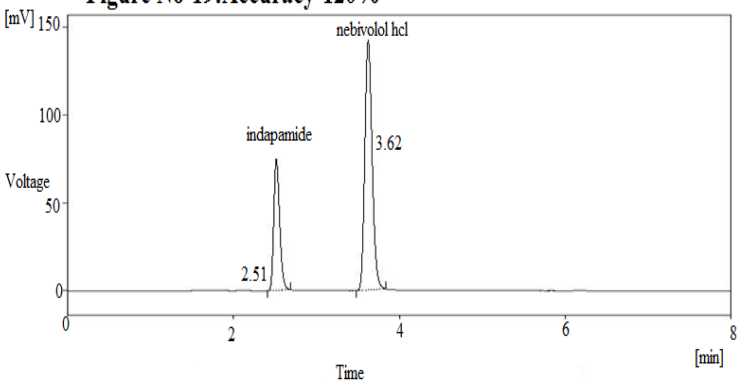


Figure No-19:Accuracy-120%



PRECISION CHROMATOGRAMS

Figure No-20:System precision-1

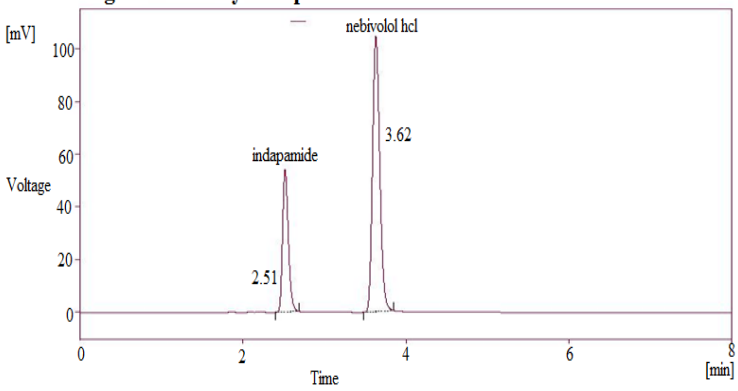


Figure No-21:System precision-2

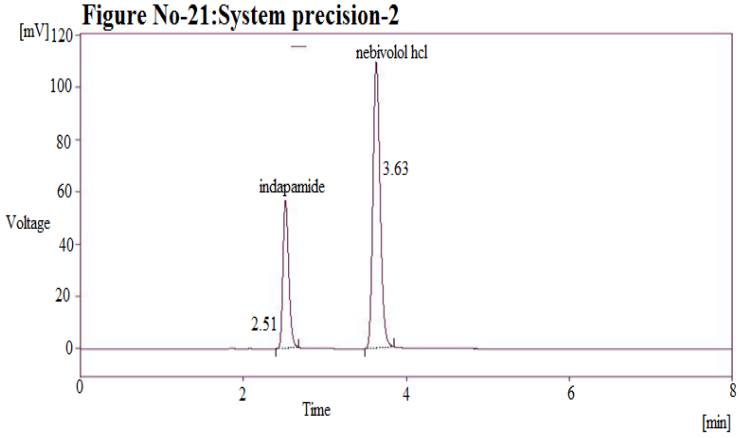


Figure No-22:System precision-3

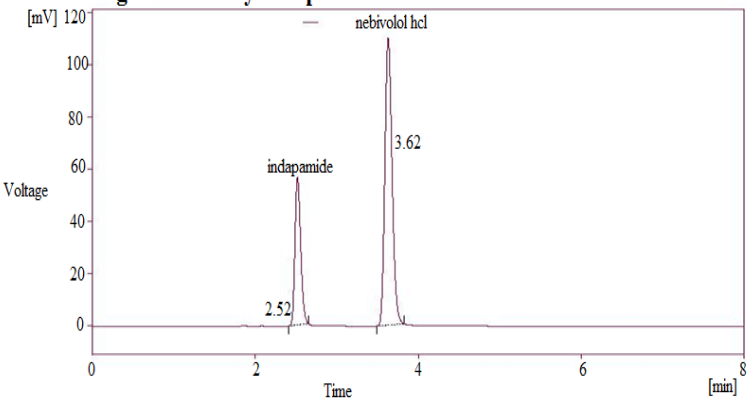


Figure No-23:System precision-4

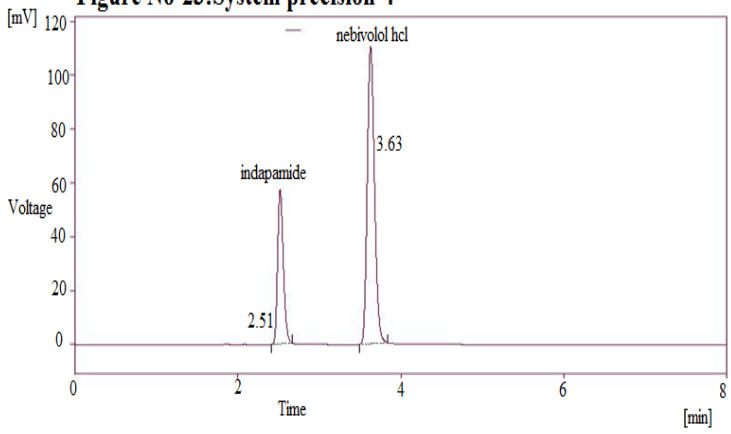


Figure No-24:System precision-5

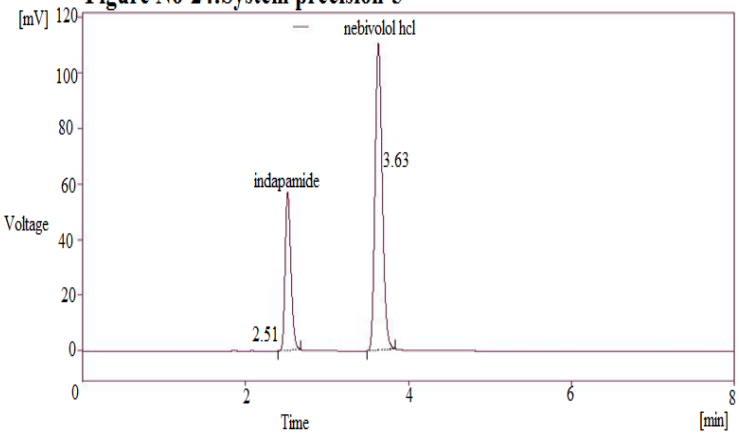


Figure No-25:System precision-6

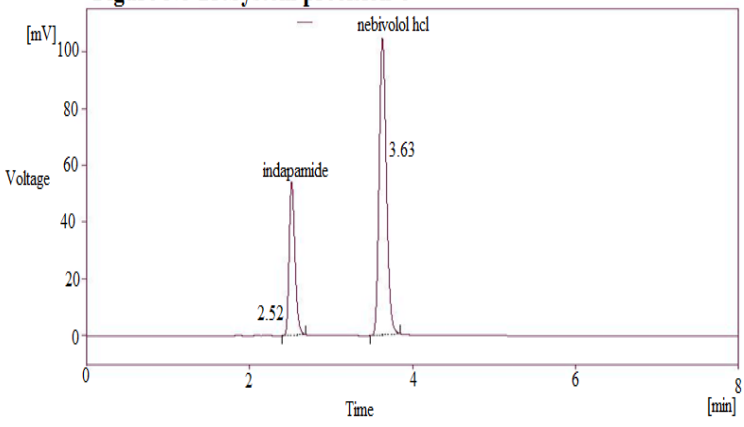


Figure No-26:Method precision-1

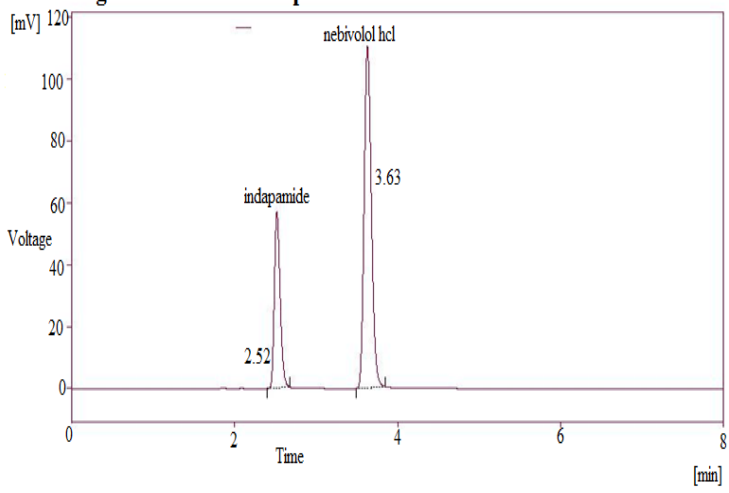


Figure No-27:Method precision-2

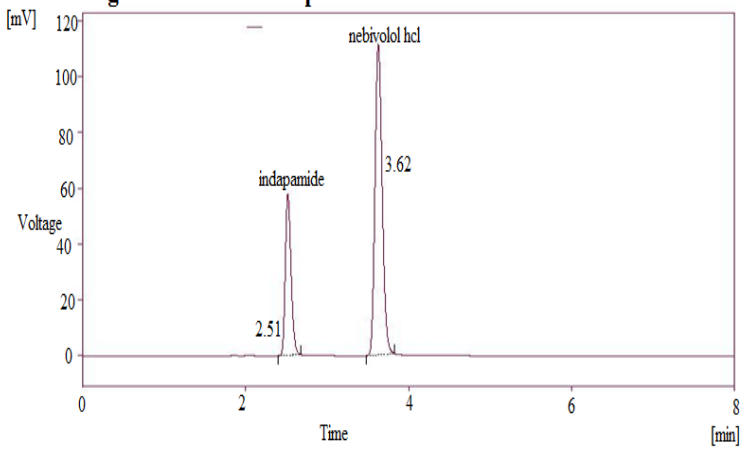


Figure No-28:Method precision-3

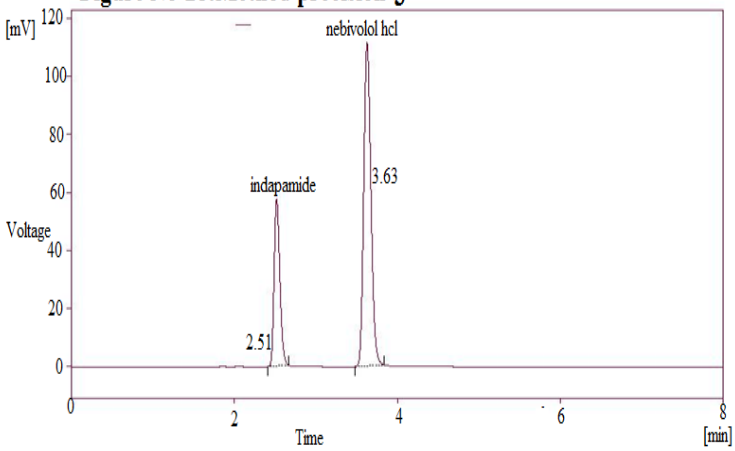


Figure No-29:Method precision-4

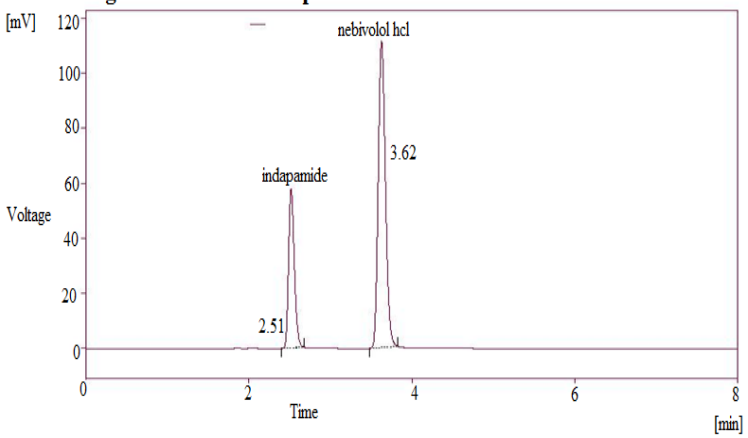


Figure No-30:Method precision-5

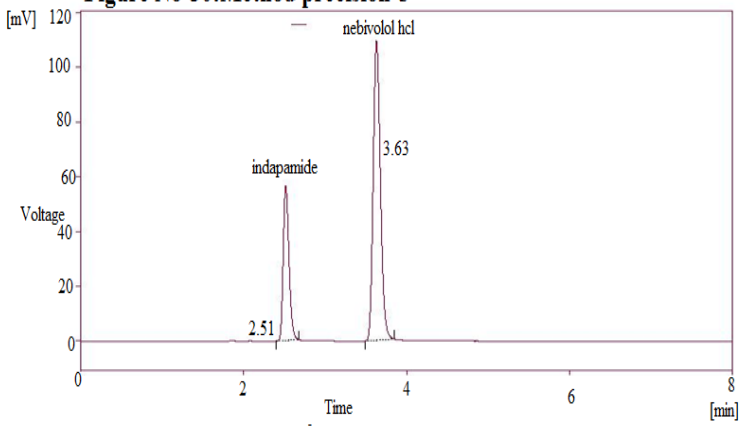
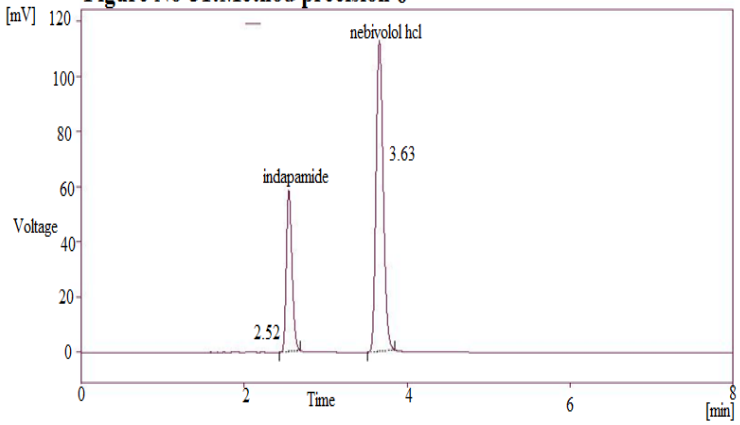


Figure No-31:Method precision-6



ROBUSTNESS CHROMATOGRAMS

Figure No-34:Robustness-1.1ml flow

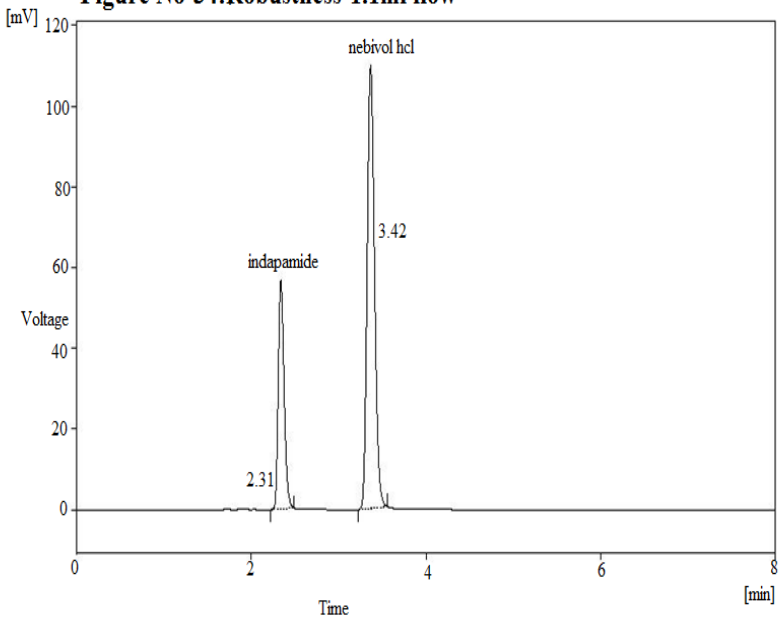
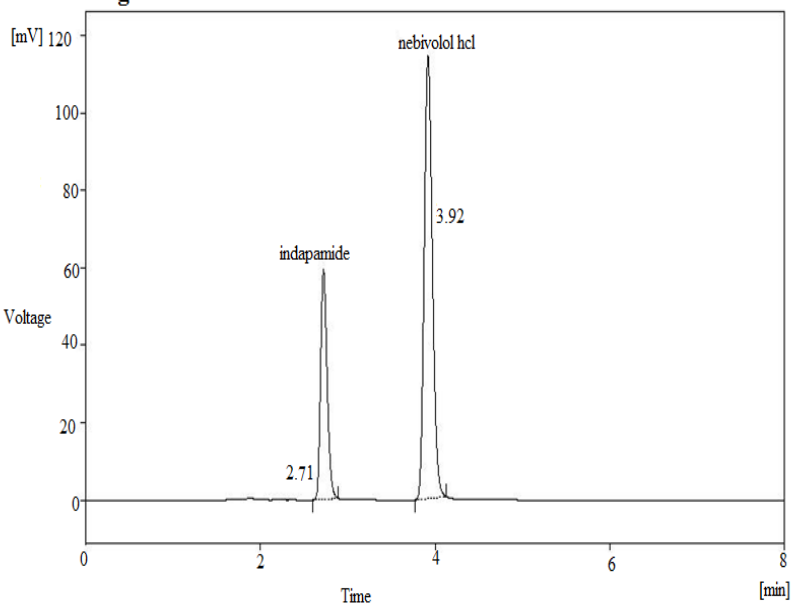


Figure No-35:Robustness-0.9ml flow



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5. Hitendra S. Joshi ,Govind J. Kher , Vijay R. Ram , Bhavesh L. Dodiya. HPLC method development and validation of combined dosage form of Atenolol and Indapamide in tablets, ***IJPT*** ,2011,Volume :3(3) , 3277-3298.

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